



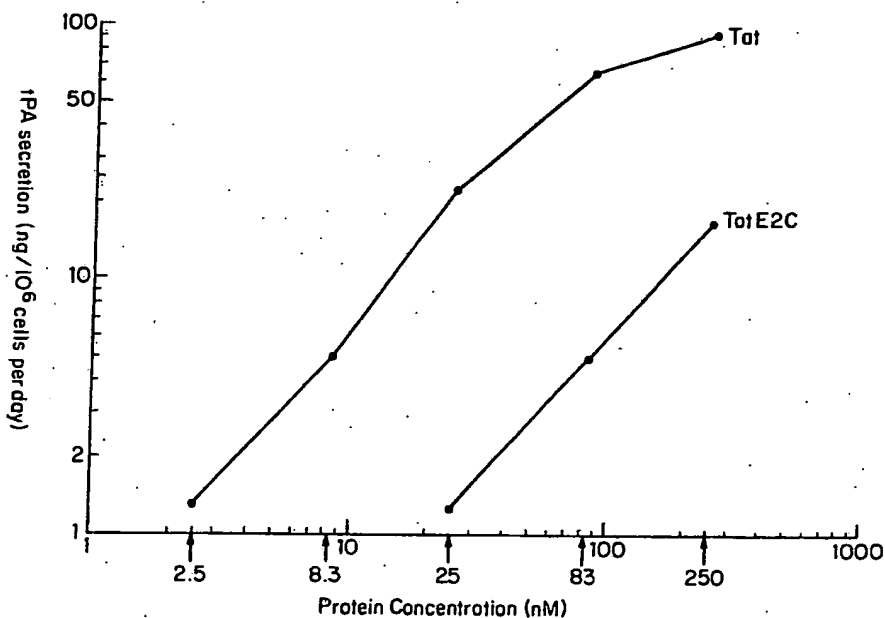
WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<p>(51) International Patent Classification ⁵ : C12N 15/87, 15/62, 15/37 A61K 47/00, 39/00</p>	<p>A2</p>	<p>(11) International Publication Number: WO 91/09958 (43) International Publication Date: 11 July 1991 (11.07.91)</p>
<p>(21) International Application Number: PCT/US90/07607 (22) International Filing Date: 21 December 1990 (21.12.90) (30) Priority data: 454,450 21 December 1989 (21.12.89) US (71) Applicants: WHITEHEAD INSTITUTE FOR BIOMEDICAL RESEARCH [US/US]; Nine Cambridge Center, Cambridge, MA 02142 (US). THE JOHNS HOPKINS UNIVERSITY SCHOOL OF MEDICINE [US/US]; 720 Rutland Avenue, Baltimore, MD 21205 (US). (72) Inventors: FRANKEL, Alan ; 3 Ellsworth Park, Cambridge, MA 02139 (US). PABO, Carl ; 120 Dunkirk Road, Baltimore, MD 21212 (US).</p>	<p>(74) Agents: GRANAHAAN, Patricia et al.; Hamilton, Brook, Smith & Reynolds, Two Militia Drive, Lexington, MA 02173 (US). (81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FI, FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), NL (European patent), NO, SE (European patent). Published <i>Without international search report and to be republished upon receipt of that report.</i></p>	

(54) Title: METHOD OF DELIVERING MOLECULES INTO EUKARYOTIC CELLS



(57) Abstract

A method of delivering a molecule of interest, which is a protein, a peptide, an oligonucleotide or a drug, into the cell, particularly into the cell nucleus and a molecule of interest-Tat protein conjugate which is useful in the method. The method and the conjugate are useful for diagnostic, prophylactic and therapeutic purposes.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	ES	Spain	MG	Madagascar
AU	Australia	FI	Finland	ML	Mali
BB	Barbados	FR	France	MN	Mongolia
BE	Belgium	GA	Gabon	MR	Mauritania
BF	Burkina Faso	GB	United Kingdom	MW	Malawi
BG	Bulgaria	GN	Guinea	NL	Netherlands
BJ	Benin	GR	Greece	NO	Norway
BR	Brazil	HU	Hungary	PL	Poland
CA	Canada	IT	Italy	RO	Romania
CF	Central African Republic	JP	Japan	SD	Sudan
CG	Congo	KP	Democratic People's Republic of Korea	SE	Sweden
CH	Switzerland			SN	Senegal
CI	Côte d'Ivoire	KR	Republic of Korea	SU	Soviet Union
CM	Cameroon	LI	Liechtenstein	TD	Chad
CS	Czechoslovakia	LK	Sri Lanka	TG	Togo
DE	Germany	LU	Luxembourg	US	United States of America
DK	Denmark	MC	Monaco		

WO 91/09958

PCT/US90/07607

-1-

METHOD OF DELIVERING MOLECULES
INTO EUKARYOTIC CELLS

Description

Background of the Invention

05 In eukaryotic cells, the nuclear envelope
isolates the central genetic processes of DNA
replication and RNA synthesis (i.e., transcription).
The cell nucleus contains nucleic acids (i.e., DNA
and RNA) and a variety of proteins. Some proteins
10 are structural proteins that bind to and organize
the DNA (e.g., histones). Other proteins are
regulating proteins that bind to DNA and thereby
positively or negatively regulate transcription
(e.g., activator and repressor proteins). Still
15 other proteins are enzymes that carry out DNA
replication and RNA synthesis (e.g., DNA and RNA
polymerase).

At the present time, there are several
techniques available for introducing molecules, such
20 as proteins and oligonucleotides, into cells. Such
techniques are of interest, for example, in genetic
engineering and as a possible means of introducing
into cells drugs or other substances potentially of
value diagnostically, therapeutically or
25 prophylactically.

Presently-available genetic engineering
transfer techniques include membrane fusion (e.g.,
fusion of cells with other cells or with liposomes),
incubation of cells with a calcium phosphate

WO 91/09958

PCT/US90/07607

-2-

precipitate of DNA fragment, DEAE-dextran-mediated transfection, electroporation, direct intracellular micro-injection of DNA fragments (e.g., via glass capillaries), and infection of cells with modified
05 vectors (e.g., viral vectors).

All of these techniques can only be used in vitro to incorporate genetic material into cells in culture. In addition, the techniques are unreliable and nonspecific. Not all cells are altered and many
10 cells do not survive the harsher treatments. Finally, these techniques are only used to transfer nucleic acids (e.g., DNA or RNA) into cells.

At the present time, there is no simple technique for delivering molecules of any type
15 (e.g., proteins or peptides, nucleic acids) directly into the nucleus of cells in vitro or in vivo. It would be very useful if it were possible to deliver such molecules reliably and specifically into the cell nucleus.

20 Summary of the Invention

The present invention pertains to the use of HIV Tat protein (Tat protein) to deliver a molecule of interest into eukaryotic cells, particularly into the cell nucleus, in vitro or in vivo. It further
25 pertains to conjugates, which include a molecule of interest and HIV Tat protein, which are useful in the method of the present invention. The method of the present invention is based on the unexpected finding that when Tat protein is present extra-
30 cellularly, it is readily taken up by cells and

WO 91/09958

PCT/US90/07607

-3-

specifically introduced into the cell nucleus, as evidenced by the fact that cells treated with Tat exhibit high levels of transactivation.

In the method of the present invention, a
05 molecule of interest and Tat protein are brought into contact with cells into which the molecule of interest is to be introduced, under conditions appropriate for its entry into cells. As a result, Tat protein and the molecule of interest enter into
10 cells, in which they pass specifically into the nucleus.

In one embodiment of the present method, a molecule of interest-Tat protein conjugate, which includes a molecule of interest (i.e., a molecule to
15 be introduced into cells) and Tat protein is brought into contact with cells into which the molecule of interest is to be introduced, under conditions appropriate for its entry into cells. As a result, the conjugate enters into cells, in which it passes
20 specifically into the nucleus.

In a further embodiment of the present method, the molecule to be delivered into cells is a protein, a peptide or an oligonucleotide. The present method is particularly useful for delivery
25 of proteins or peptides, such as regulatory factors, enzymes, antibodies, drugs or toxins, as well as DNA or RNA, into the cell nucleus.

A stabilizing agent, which serves to increase Tat stability and uptake, can be brought into
30 contact with cells, in conjunction with the molecule of interest and Tat protein. For example, metal

WO 91/09958

PCT/US90/07607

-4-

ions which bind to Tat protein and increase its stability and uptake, can be used for this purpose.

In a further embodiment, a lysosomotropic agent is provided extracellularly in conjunction
05 with Tat protein and a molecule of interest, in order to enhance uptake by cells. The lysosomotropic agent can be used alone or in conjunction with a stabilizer. For example, lysosomotropic agents
10 such as chloroquine, monensin, amantadine and methylamine which have been shown to increase uptake of Tat in some cells by a few hundred fold, can be used for this purpose.

In another embodiment, a basic peptide, such as Tat 38-58 or protamine, is provided extracellularly
15 with Tat and a molecule of interest to enhance uptake of Tat. Such basic peptides can also be used alone, in combination or with stabilizing agents or lysosomotropic agents.

Through use of the present method, it is
20 possible to introduce into cells and, particularly into the cell nucleus, a drug or other substance which is of diagnostic, therapeutic or prophylactic value.

Brief Description of the Drawings

25 Figure 1 is the amino acid sequence of the HIV-1 Tat protein.

Figure 2 is a thin layer chromatogram (TLC) showing chloramphenicol acetyl transferase (CAT) activity, a measure of Tat uptake, resulting from

WO 91/09958

PCT/US90/07607

-5-

incubating HL3T1 cells with Tat for 24 hours at the indicated concentrations.

Figure 3 is a TLC showing CAT activity resulting from incubating HL3T1 cells with 5 μ g of Tat and a variety of lysosomotropic agents.

Figure 4 is a graph showing the cellular uptake and nuclear localization of 125 I-labeled Tat.

Figure 5 is a gel of nuclear fractions from HL3T1 cells treated with Tat protein in the absence (-) or presence (+) of chloroquine.

Figure 6 is a series of graphic representations showing chloroquine stimulation of Tat transactivation.

Figure 6A is a graph showing the effect of chloroquine concentration on uptake and transactivation by 2 μ g Tat added to the medium.

Figure 6B is a graph showing the time course of uptake and transactivation by 2 μ g of Tat and 100 μ M chloroquine.

Figure 6C is a graph showing the effect on uptake and transactivation by several concentrations of Tat with 100 μ M chloroquine.

Figure 7 is a TLC showing CAT activity from H9 lymphocytes, U937 promonocytes and HeLa cells treated with Tat protein in the absence or presence of chloroquine.

Figure 8 is a TLC showing CAT activity from HL3T1 cells and illustrating the extent of activation of the CAT reporter gene following various time periods of exposure to 1 μ g Tat + 100 μ M chloroquine.

WO 91/09958

PCT/US90/07607

-6-

Figure 9 is a schematic representation of the murine sarcoma virus (MSV) retroviral vector used to establish the H938 reporter cell line from H9 cells. The transcription start sites from the SV40 promoter, the HIV and MSV LTRs are indicated by arrows, and the location and size of the fragments protected in the RNase analysis are indicated by bars.

Figure 10 illustrates the results of an RNase protection experiment in H938 cells, using an α -³²P UTP-labeled HIV-1 LTR probe corresponding to a 200 bp fragment from -120 to +80 of the viral LTR, prepared by in vitro transcription.

Figure 11 is a graph illustrating the enhancement of transactivation in H938 cells upon addition of increasing amounts of the Tat 38-58 peptide with 1 μ g of Tat as assayed by CAT activity after a 24 hour incubation with peptide and Tat.

Figure 12 is a graph illustrating transactivation of a tPA reporter gene under the control of the HIV-1 LTR in HeLa.318 cells upon addition of exogenous Tat or a Tate2C fusion protein.

Detailed Description of the Invention

The present invention is based on the unexpected finding that Tat protein from immunodeficiency virus (e.g., HIV-1, HIV-2, SIV) is readily taken up into cells and subsequently into the cell nucleus. Tat is a potent viral transactivator and is essential for viral replication. In light of the

WO 91/09958

PCT/US90/07607

-7-

fact that proteins and peptides are typically poorly taken up (Sternson, L.A., Ann. N.Y. Acad. Sci. 57:19-21 (1987)), the finding that Tat is readily taken up into cells is surprising.

05 As a result of this finding, it is now possible to use Tat protein to deliver molecules (e.g., proteins, peptides, nucleic acids) into cells and, specifically, into the cell nucleus. The present invention is a method of delivering a molecule of
10 interest into cells and, particularly, of targeting a molecule to the cell nucleus, as well as a conjugate useful in the method. Any molecule can be delivered into cells, especially into the cell nucleus, using the method of the subject invention.

15 In one embodiment of the method of the present invention, a molecule of interest-Tat protein conjugate, which includes a molecule of interest (i.e., a molecule to be introduced into cells and delivered to the nucleus) attached to HIV Tat
20 protein, is brought into contact with cells into which introduction of the molecule of interest is desired. In another embodiment, a molecule of interest and HIV Tat protein are contacted with cells into which the molecule of interest is to be
25 introduced. The method can be used to deliver a molecule of interest either in vitro or in vivo. For example, delivery can be carried out in vitro by adding a molecule of interest-Tat conjugate to cultured cells, by producing cells that synthesize
30 Tat or Tat conjugate or by combining a sample (e.g., blood, bone marrow) obtained from an individual with

WO 91/09958

PCT/US90/07607

-8-

the conjugate, under appropriate conditions.
Delivery can be carried out in vivo by administering
the molecule of interest and Tat protein to an
individual in whom it is to be used for diagnostic,
05 preventative or therapeutic purposes.

The following is a description of: the HIV Tat
protein; uptake of Tat into the cell nucleus; the
molecule of interest-Tat protein conjugate; and the
method by which the conjugate is used to deliver a
10 selected substance into cells.

HIV Tat protein

Figure 1 shows the amino acid sequence of the
Tat protein. The full-length Tat protein is 86
amino acids long and is encoded by two exons; the
15 N-terminal 72 residues are encoded by the first exon
and the C-terminal 14 residues are encoded by the
second exon. Tat protein contains a highly basic
region (with 2 lysines and 6 arginines in 9
residues) and a cysteine-rich region (with 7
20 cysteines in 16 residues).

The basic region (i.e., amino acids 49-57) is
thought to be important for nuclear localization.
Ruben, S. et al., J. Virol. 63:1-8 (1989); Hauber,
J. et al., J. Virol. 63:1181-1187 (1989). The
25 cysteine-rich region mediates the formation of
metal-linked dimers in vitro (Frankel, A.D. et al.,
Science 240:70-73 (1988)); (Frankel, A.D. et al.,
Proc. Natl. Acad. Sci, USA, 85:6297-6300 (1988));
and is essential for its activity as a
30 transactivator (Garcia, J.A. et al., EMBO J. 7:3143

WO 91/09958

PCT/US90/07607

-9-

(1988); Sadaie, M.R. et al., J. Virol. 63:1 (1989)).

Like other regulatory proteins, the N-terminal region may be involved in protection against intracellular proteases (Bachmair, A. and A.

05 Varshavsky, Cell, 56:1019-1032 (1989)).

It will be appreciated that the entire 86 amino acids which make up the Tat protein may not be required for the uptake activity of Tat. For

example, a protein fragment or a peptide which has
10 fewer than the 86 amino acids, but which exhibits uptake into cells and uptake into the cell nucleus, can be used (a functionally effective fragment or

portion of Tat). As is shown in the Examples below, Tat protein containing residues 1-72 is sufficient

15 for uptake activity and Tat residues 1-67 are shown to mediate the entry of a heterologous protein into cells. In addition, a synthetic peptide containing Tat residues 1-58 has now been shown to have uptake activity. A Tat peptide comprising the region that
20 mediates entry and uptake into cells can be further defined using known techniques. (see, e.g.,

Frankel, A.D., et al., Proc. Natl. Acad. Sci, USA, 86: 7397-7401 (1989)).

The Tat peptide can be a single (i.e., continu-
25 ous) amino acid sequence present in Tat protein or it can be two or more amino acid sequences which are present in Tat protein, but in the naturally-occurring protein are separated by other amino acid sequences. As used herein, Tat protein includes a
30 naturally-occurring amino acid sequence which is the same as that of naturally-occurring Tat protein, its

WO 91/09958

PCT/US90/07607

-10-

functional equivalent or functionally equivalent fragments thereof (peptides). Such functional equivalents or functionally equivalent fragments possess uptake activity into the cell and into the cell nucleus that is substantially similar to that of naturally-occurring Tat protein. Tat protein can be obtained from naturally-occurring sources or can be produced using genetic engineering techniques or chemical synthesis.

The amino acid sequence of naturally-occurring HIV Tat protein can be modified, by addition, deletion and/or substitution of at least one amino acid present in the naturally-occurring Tat protein, to produce modified Tat protein (also referred to herein as Tat protein). Modified Tat protein or Tat peptide analogs with increased stability can thus be produced using known techniques. Therefore, Tat proteins or peptides may have amino acid sequences which are substantially similar, although not identical, to that of naturally-occurring Tat protein or portions thereof. In addition, cholesterol or other lipid derivatives can be added to Tat protein to produce a modified Tat having increased membrane solubility.

Variants of Tat protein can be designed to modulate the intracellular location of Tat and the molecule of interest following uptake into the cell or when expressed in the cell. When added exogenously, such variants are designed such that the ability of Tat to enter cells is retained (i.e., the uptake of the variant Tat protein or peptide

WO 91/09958

PCT/US90/07607

-11-

into the cell is substantially similar to that of naturally-occurring HIV Tat). For example, alteration of the basic region thought to be important for nuclear localization (see e.g., Dang, C.V. and Lee, W.M.F., J. Biol. Chem. 264: 18019-18023 (1989); Hauber, J. et al., J. Virol. 63: 1181-1187 (1989); Ruben, S.A. et al., J. Virol. 63: 1-8 (1989)) can result in a cytoplasmic location or partially cytoplasmic location of Tat, and therefore, of the molecule of interest. Alternatively, a sequence for binding a cytoplasmic component can be introduced into Tat in order to retain Tat and the molecule of interest in the cytoplasm or to confer regulation upon nuclear uptake of Tat and the molecule of interest.

Demonstration of Uptake of Tat into the Cell Nucleus

In an attempt to develop a convenient assay for the Tat protein, various ways of introducing Tat protein into cells containing a reporter gene (i.e., a gene that can be transcribed to express an assayable protein, e.g., the chloroamphenicol acetyl-transferase or CAT gene) were tried. Scrape loading, as described by McNeil and co-workers, was shown to be a convenient method which gave quantitative and reproducible transactivation of the HIV-1 promoter (McNeil, P.L. et al., J. Cell Biol., 98:1556-1564 (1984)). It is believed that scrape-loading transiently damages the cell membrane and allows molecules present in the culture medium to equilibrate with the cytoplasm. An unexpected

WO 91/09958

PCT/US90/07607

-12-

result was seen when Tat was simply added to the culture medium of HL3T1 cells (HeLa cells containing the integrated LTR-CAT plasmid): Expression of CAT from the integrated HIV-1 promoter increased and was proportional to the Tat concentration, indicating that Tat was taken up and transactivated the HIV-1 promoter. This result was surprising because proteins and peptides are generally believed to be poorly taken up by cells. Sternson, L.A., Ann. N.Y. Acad. Sci., 5719-21 (1987).

To measure cellular uptake directly, HL3T1 cells were treated with ^{125}I -labeled Tat in the presence or absence of chloroquine, and the amount of radioactive Tat present in various cellular fractions was determined. This work is described in greater detail in Example III.

Figure 2 shows results of assessment of the expression of CAT by HL3T1 cells incubated with Tat protein for 24 hours, at the indicated concentrations. Expression of CAT from the integrated HIV-1 promoter increased and was proportional to the Tat concentration. CAT activity did not increase further after 24 hours. Additional small increases in activity (2- to 3-fold) were observed upon addition of 10 mM zinc or 1 mM cadmium, suggesting that metals might stabilize Tat either during uptake or once inside the cell.

To explore the uptake process further, various lysosomotropic agents were added to the culture medium. Lysosomotropic agents are thought to

WO 91/09958

PCT/US90/07607

-13-

inhibit receptor-mediated endocytosis. Mellman, I. et al., Ann. Rev. Biochem., 55:663-700 (1986).

Figure 3 shows the effect that a variety of lysosomotropic agents have on uptake and subsequent transactivation by Tat placed in tissue culture medium. HL3T1 cells were incubated with 5 μ g of Tat (100 nM) and each agent for 24 hours, the medium was replaced, and CAT activity was determined after 60 hours. Activity from untreated cells, cells incubated with Tat alone and cells incubated with chloroquine alone are also shown. The level of uptake and subsequent transactivation in HL3T1 cells by 5 μ g of Tat with chloroquine present was about 7000-fold compared with untreated cells, whereas chloroquine gave little increase in promoter activity in the absence of Tat. Monensin, amantadine and methylamine also significantly increased transactivation, whereas ammonium chloride only slightly increased activity. No lysosomotropic agent tested significantly activated the promoter in the absence of Tat. The parameters of chloroquine-stimulated Tat activity are explained in more detail in Example IV.

Figure 4 shows that within 6 hours after treating HL3T1 cells with Tat and chloroquine, a significant amount of radioactive Tat (about 3% of the total) had been taken up by the cells. Most of this Tat (<80%) was localized in the nuclear fraction. Trypsin-sensitive counts, representing Tat bound to the cell surface, remained relatively

WO 91/09958

PCT/US90/07607

-14-

constant and by 12 hours were less than 20% of the counts found in the nucleus.

Figure 5 shows nuclear extracts run on an SDS gel. A radioactive band comigrating with intact Tat is readily apparent. HL3T1 cells treated with Tat but without chloroquine showed similar kinetics of uptake and nuclear localization when assayed by counting the cellular fractions but only degraded Tat was seen on the gel.

The ability of Tat to directly enter lymphocytes or monocytes was also assessed; Tat readily entered both types of cells, as demonstrated by the high levels of transactivation in cells treated with Tat, alone or with chloroquine. H9 lymphocytes and U937 promonocytes (10^6 cells) containing an integrated HIV-1 LTR-CAT plasmid (H938 and U38 cells, respectively (Felber, B.K. and G.N. Pavlakis, Science, 239:184 (1988)) were incubated in RPMI 1640 medium containing 10% fetal bovine serum (1 ml in 25 mm wells) at 37°C (no tat), treated with 5 μ g of Tat protein (tat) or treated with 5 μ g of Tat and 100 μ m chloroquine (tat + CQ). Cells were harvested 24 hours after Tat treatment and assayed for CAT activity (Gorman, C.M. et al., Mol. Cell Biol., 2:1044 (1982). HeLa cells (10^6 cells) containing an integrated HIV-1 LTR-CAT plasmid (HL3T1) (Felber, B.K. and G.N. Pavlakis, Science, 239:184 (1988)), were incubated in Delbucco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (1 ml in 25 mm wells) and similarly treated with Tat protein, or with Tat and chloroquine, and assayed for CAT

WO 91/09958

PCT/US90/07607

-15-

activity. Unacetylated (cm) and acetylated (ac) forms of ^{14}C chloramphenicol were separated by thin layer chromatography.

Figure 7 shows the results of this assessment of Tat entry into lymphocytes and monocytes. High levels of transactivation were seen in all three cell lines. In the HeLa cells, the addition of chloroquine resulted in a significant stimulation of Tat activity. However, in contrast to the case with HeLa cells, chloroquine had little effect on Tat entry into lymphocytes or monocytes. The chloroquine-independent entry into lymphocytes and monocytes may suggest a different mechanism of uptake.

The time course of binding was determined in HeLa cells containing an integrated HIV-1 LTR-CAT plasmid (HL3T1 cells) (Felber, B.K. and G.N. Pavlakis, Science, 239:184 (1988)). Cells (2×10^6) were grown to confluence in 12 well tissue culture plates (12mm well diameter), and washed with phosphate buffered saline (PBS). Cells were incubated in fresh DMEM with $1 \mu\text{g}$ Tat (1-72) and $100 \mu\text{M}$ chloroquine at 37°C for different lengths of time. Following two washes with PBS to remove Tat, fresh medium was added and transactivation was measured 24 hours after Tat addition. CAT activity was used as a measure of transactivation.

The results of this analysis are shown in Figure 8. The basal level of expression from the HIV-1 LTR in the absence of Tat is shown in the "no Tat" lane. Maximal levels of transactivation were

WO 91/09958

PCT/US90/07607

-16-

observed after a five minute exposure to Tat. Thus, binding is rapid, and a brief exposure can result in uptake by cells, as assayed by transactivation.

The time required to observe a response to
05 exogenous Tat was determined in H938 cells. H938 cells were derived from the H9 lymphoid cell line by infection with a murine sarcoma virus (MSV) retroviral vector. (Felber, B.K. and G.N. Pavlakis, Science, 239:184 (1988)). The integrated MSV vector
10 contains the CAT gene under the control of the HIV-1 LTR, and the neo gene under the control of an SV40 promoter (Figure 9). H938 cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, penicillin (250 U/ml), and streptomycin (250
15 µg/ml). The cells were treated with 10 µg/ml of Tat protein (amino acids 1-72) in the presence of 100 µg/ml protamine, and RNA was prepared and analyzed by RNase protection. An α -³²P UTP-labeled HIV-1 LTR probe corresponding to a 200 bp fragment from -120
20 to +80 of the viral LTR was prepared by in vitro transcription. These procedures are further described in Example V.

The results of the RNase protection assay are shown in Figure 10. Two major fragments were
25 protected. The 80 nucleotide fragment is derived from transcripts expressed from the HIV LTR and the 200 nucleotide fragment is derived from transcripts expressed from either the upstream MSV or SV40 promoters. Transcription from the HIV LTR increased
30 after 15 minutes of exposure to Tat, and reached a maximum by 2-6 hours. In contrast, transcription

WO 91/09958

PCT/US90/07607

-17-

from the upstream MSV and SV40 promoters was not increased by Tat addition, indicating that exogenously added Tat retains specificity for the HIV promoter. When Tat protein is added exogenously to cells, there is a significant increase in transcription in 15 minutes, indicating that Tat can enter cells, become localized to the nucleus, bind to its target site TAR specifically, and promote transcription within 15 minutes. (The short transcripts, which may be degradation products from incompletely elongated RNAs, were not affected by Tat.)

Several peptide fragments of Tat were tested for their ability to compete for Tat binding and uptake in HL3T1 and H938 cells. In these experiments, 0.5×10^6 H938 cells were pelleted and resuspended in 0.5 ml fresh RPMI 1640 medium. Cells were incubated at 37 °C with 1 µg Tat (1-72) and increasing concentrations of peptide. Extracts were prepared after 24 hours and assayed for CAT activity. Surprisingly, Tat 38-58, which contains the basic region of Tat, actually enhanced the effect of exogenous Tat and increased transactivation in a concentration dependent manner. Figure 11 shows the results of this experiment in the H938 cell line. The data was quantitated by cutting the spots from the TLC plates and counting the associated radioactivity in a scintillation counter.

Protamine (protamine sulfate, Sigma), another basic peptide, was also observed to enhance

WO 91/09958

PCT/US90/07607

-18-

transactivation by extracellular Tat when present at a concentration of 100 μ g/ml. However, a smaller Tat peptide, containing only the basic region from 47-58, had no effect on transactivation under the conditions used. A mixture of two peptides, Tat 05 38-47 and Tat 48-58, the products of chymotryptic digestion of Tat 38-58, also had no effect on transactivation under these conditions. No enhancement of activity by protamine was seen when HL3T1 cells were scrape-loaded with Tat, suggesting 10 that protamine directly affects the uptake process.

Other cell lines were also tested for Tat uptake and transactivation activity. Jurkat T cells showed significant transactivation when Tat was added to the medium and showed further 15 transactivation in the presence of chloroquine. A Vero line (VNHIV-CAT; Mosca, J.D. *et al.*, Nature, 325:67-70 (1987)) also showed significant transactivation upon incubation of cells with Tat and chloroquine; no activity was seen with Tat 20 alone. However, since the basal expression of CAT was low in this cell line, a several fold increase in CAT activity would still have been undetectable.

To directly follow the entry of Tat into live cells, Tat was labelled with rhodamine (TRITC-Tat) 25 and its movement was followed by fluorescence microscopy. Punctate staining was observed on the surface of HL3T1 and H938 cells immediately after incubation with TRITC-Tat, similar to that seen in receptor-mediated endocytosis. After one hour, 30 clear nuclear staining was observed in HL3T1 cells.

WO 91/09958

PCT/US90/07607

-19-

Punctate cytoplasmic staining was also observed, suggesting that Tat may be localized within endosomes. Incubation at low temperature, which blocks endocytosis, also blocked entry of
05 rhodamine-labeled Tat. After six hours, most of the Tat was in the nucleus of HL3T1 cells, but was excluded from the nucleoli. Remarkably, every cell in the culture was labeled with TRITC-Tat, indicating that the uptake of exogenous Tat is
10 efficient. (Cellular localization was also examined in H938 cells, however, since the nucleus constitutes most of the lymphocytic cell, it was difficult to distinguish nuclear from non-nuclear compartments.) When tested for transactivation,
15 TRITC-Tat was found to have the same specific activity as unmodified Tat.

Tat-mediated uptake of a heterologous protein

A preliminary assessment of the ability of Tat
20 to mediate the uptake of a molecule of interest was carried out. Additional details of this analysis are provided in Example VII. The E2 open reading frame of the bovine papillomavirus-1 (BPV-1; Chen, E.Y. et al., Nature 299: 529-534 (1982)) encodes
25 both positive and negative acting transcriptional regulators (regulatory factors; Sousa, R. et al., Biochim. Biophys. Acta 1032: 19-37 (1990); Lambert, P.F et al., J. Virol. 63(7): 3151-3154 (1989);
Lambert, P.F. et al., Cell 50: 69-78 (1987)). A
30 fusion gene was constructed in which the HIV-1 tat gene was linked to the carboxy-terminal region of

WO 91/09958

PCT/US90/07607

-20-

the E2 open reading frame. The construct which encodes the fusion protein, pFTE103 (constructed by Dr. J. Barsoum, Biogen, Inc.), was designed to express a protein comprising amino acids 1 through 67 of Tat at the amino terminus, followed by the C-terminal 105 amino acids of E2 (residues 306 through 410 of BPV-1 E2), which contain the DNA binding domain of the E2 open reading frame (EP 0,302,758, Androphy et al., (Feb. 6, 1989); Giri, I. and Yaniv, M., EMBO J., 7(9): 2823-2829 (1988); McBride, A.A. et al., EMBO J. 7(2): 533-539 (1988); Androphy, E.J. et al., Nature 325: 70-73 (1987)). pFTE103 was introduced into E. coli and the TatE2C fusion protein was expressed using the T7 RNA polymerase expression system as described by Studier et al. (Studier et al., Methods in Enzymology 185:60-89 (1990)). The purified TatE2C fusion protein migrated with an apparent molecular weight of 20,000 to 21,000 daltons on protein gels. Uptake of the TatE2C fusion protein was tested following introduction into the culture medium of animal cells.

Uptake of the Tat portion of the fusion protein (molecule of interest-Tat protein conjugate) was assayed by measuring transactivation of a Tat-responsive reporter construct integrated into HeLa cells (HeLa.318 cells). The Tat-responsive reporter construct (pXB318) present in HeLa.318 cells contains the human tissue plasminogen activator (tPA) cDNA reporter gene from pTPA114 (Fisher et al. J. Biol. Chem. 260:11223-11230

WO 91/09958

PCT/US90/07607

-21-

(1985)) under the control of the HIV-1 long terminal repeat (LTR) from pU3R-III (Sodroski et al. Science 227:171-173 (1985)). Tat protein (amino acids 1-72) or the TatE2C fusion protein were added to the
05 culture medium of HeLa.318 cells in 24 well plates at concentrations ranging from 2.5 nM to 250 nM, in the presence of 100 μ M chloroquine, essentially as described (Frankel, A.D. and Pabo, C.O., Cell 55:1189-1193 (1988)). The culture medium was
10 harvested 24 hours later and assayed for tPA activity by the method of Granelli-Piperno and Reich (J. Exp. Med. 148:223-234 (1978)). Cell numbers were determined and tPA secretion was expressed as ng/10⁶ cells per day. Figure 12 shows the results
15 obtained from a tPA assay of HeLa.318 media 24 hours after the addition of Tat or TatE2C protein to culture medium. In the absence of Tat or the TatE2C protein, tPA activity was undetectable (less than 0.1 ng/10⁶ cells per day). However, addition of
20 either Tat or TatE2C protein led to an increase in tPA production (Figure 12). Thus, it appears that Tat (residues 1-67) can retain the ability to enter cells when linked to a heterologous protein.

Although transactivation upon addition of the
25 TatE2C protein was somewhat less efficient than that observed upon addition of Tat, the TatE2C fusion protein was also less active than Tat in transactivation assays when the proteins were produced intracellularly after transfection of the
30 genes into HeLa.318 cells. Thus, it is not clear whether the apparent reduction in activity is

WO 91/09958

PCT/US90/07607

-22-

attributable to reduced uptake or reduced activity of the fusion protein produced by E. coli and added exogenously. It is possible that some Tat activity may be lost during the denaturation and refolding of the TatE2C fusion protein during purification.

Uptake of the E2 portion of the conjugate was determined by indirect immunofluorescence using rabbit polyclonal serum raised against E2-C85 (the C-terminal 85 amino acids of the E2 protein produced in E. coli). For indirect immunofluorescence, mouse 3T3 cells were seeded into LAB-TEK four chamber tissue culture chamber/slides. The next day, TatE2C fusion protein was added at 250 nM to the culture medium, in the presence of 100 μ M chloroquine. Six hours later, immunofluorescence was performed as described in Example VII.

While only very faint background fluorescence was seen when E2.C85 protein was added to cells (at the same concentration and in the presence of 100 μ M chloroquine), addition of the TatE2C fusion protein led to very intense fluorescence in all cells observed. These cells displayed fluorescence on the plasma membrane, in the cytosol and in nuclei. The staining was present in bright patches rather than evenly dispersed throughout the cells. The amount of E2 fluorescence obtained following addition of TatE2C protein to culture medium was far greater than the immunofluorescence observed when a TatE2C gene was expressed in these same cells. These data indicate that the Tat protein is capable of efficiently carrying a heterologous protein present

WO 91/09958

PCT/US90/07607

-23-

as part of a molecule of interest-Tat conjugate into cells.

The molecule of interest-Tat protein conjugate

05 A molecule of interest, which will generally be
a protein or peptide, a nucleotide sequence, or
other chemical which has diagnostic, prophylactic or
therapeutic application (referred to herein as a
drug) is combined, as described below, with HIV Tat
protein to produce a molecule of interest-Tat
10 protein conjugate the resulting conjugate is brought
into contact with the extracellular surface of
cells.

In one embodiment of the present invention, the
molecule of interest is a protein, such as an
15 enzyme, antibody, toxin, or regulatory factor (e.g.,
transcription factor) whose delivery into cells, and
particularly into the cell nucleus is desired. For
example, some viral oncogenes inappropriately turn
on expression of cellular genes by binding to their
20 promoters. By providing a competing binding protein
in the cell nucleus, viral oncogene-activity can be
inhibited.

In a further embodiment, the molecule of
interest is a nucleotide sequence to be used as a
25 diagnostic tool (or probe), or as a therapeutic
agent, such as an oligonucleotide sequence which is
complementary to a target cellular gene or gene
region and capable of inhibiting activity of the
cellular gene or gene region by hybridizing with it.
30 In yet another embodiment, the molecule of interest

WO 91/09958

PCT/US90/07607

-24-

is a drug, such as a peptide analog or small molecule enzyme inhibitor, whose introduction specifically and reliably into the cell nucleus is desired.

05 The molecule of interest can be obtained or produced using known techniques, such as chemical synthesis, genetic engineering methods and isolation from sources in which it occurs naturally. The molecule of interest can be combined with or
10 attached to the Tat protein to form the molecule of interest-Tat protein conjugate which is a subject of the present invention.

 The attachment of the molecule of interest to Tat to produce a molecule of interest-Tat protein
15 conjugate may be effected by any means which produces a link between the two constituents which is sufficiently stable to withstand the conditions used and which does not alter the function of either constituent. Preferably, the link between them is
20 covalent. For example, recombinant techniques can be used to covalently attach Tat protein to molecules, such as by joining the gene coding for the molecule of interest with the gene coding for Tat and introducing the resulting gene construct
25 into a cell capable of expressing the conjugate. Alternatively, the two separate nucleotide sequences can be expressed in a cell or can be synthesized chemically and subsequently joined, using known techniques. Alternatively, the protein of
30 interest-Tat molecule can be synthesized chemically as a single amino acid sequence (i.e., one in which

WO 91/09958

PCT/US90/07607

-25-

both constituents are present) and, thus, joining is not needed.

Coupling of the two constituents can be accomplished via a coupling or conjugating agent.

- 05 There are several intermolecular cross-linking reagents which can be utilized (see, for example, Means, G.E. and Feeney, R.E., Chemical Modification of Proteins, Holden-Day, 1974, pp. 39-43). Among these reagents are, for example, J-succinimidyl
- 10 3-(2-pyridyldithio) propionate (SPDP) or N, N'-(1,3-phenylene) bismaleimide (both of which are highly specific for sulhydryl groups and form irreversible linkages); N, N'-ethylene-bis-(iodoacetamide) or other such reagent having 6 to 11
- 15 carbon methylene bridges (which relatively specific for sulhydryl groups); and 1,5-difluoro-2,4-dinitrobenzene (which forms irreversible linkages with amino and tyrosine groups). Other cross-linking reagents useful for this purpose include: p,p'-di-
- 20 fluoro-m, m'-dinitrodiphenylsulfone (which forms irreversible cross-linkages with amino and phenolic groups); dimethyl adipimidate (which is specific for amino groups); phenol-1,4-disulfonylchloride (which reacts principally with amino groups); hexamethylenediisocyanate or diisothiocyanate, or azophenyl-
- 25 p-diisocyanate (which reacts principally with amino groups); glutaraldehyde (which reacts with several different side chains) and disdiazobenzidine (which reacts primarily with tyrosine and histidine).

WO 91/09958

PCT/US90/07607

-26-

Delivery of a molecule of interest using the present method

The present method can be used to deliver a molecule of interest into cells, particularly into the cell nucleus, in vitro or in vivo. In in vitro applications in which the molecule is to be delivered into cells in culture, the molecule of interest in combination with Tat protein or the molecule of interest-Tat protein conjugate is simply added to the culture medium. This is useful, for example, as a means of delivering into the nucleus substances whose effect on cell function is to be assessed. For example, the activity of purified transcription factors can be measured, or the in vitro assay can be used to provide an important test of a molecule's activity, prior to its use in in vivo treatment.

Alternatively, the molecule of interest in combination with Tat protein or the molecule of interest-Tat protein conjugate can be used for prophylactic or therapeutic purposes (for the treatment, prophylaxis or diagnosis of a disease or condition). For example, a selected molecule of interest in combination with Tat protein or the molecule of interest-Tat protein conjugate can be combined with a sample obtained from an individual (e.g., blood, bone marrow) in order to introduce the molecule of interest into cells present in the sample and, after treatment in this manner, the sample returned to the individual. A series of treatments carried out in this manner can be used to

WO 91/09958

PCT/US90/07607

-27-

prevent or inhibit the effects of an infectious agent. For example, blood can be removed from an individual infected with HIV or other viruses, or from an individual with a genetic defect. The blood
05 can then be combined with a molecule of interest in combination with Tat protein or a molecule of interest-Tat protein conjugate in which the molecule of interest is a drug capable of inactivating the virus or an oligonucleotide sequence capable of
10 hybridizing to a selected virus sequence and inactivating it or a protein that supplements a missing or defective protein, under conditions appropriate for entry in cells of the conjugate and maintenance of the sample in such a condition that
15 it can be returned to the individual. After treatment, the blood is returned to the individual.

Alternatively, the molecule of interest in combination with Tat protein or a molecule of interest-Tat protein conjugate can be delivered in
20 vivo. For example, cells that synthesize Tat or Tat conjugate can be produced and implanted into an individual so that Tat or Tat conjugate is constantly present. In another embodiment, the conjugate can be used much like a conventional
25 therapeutic agent and can be a component of a pharmaceutical composition which includes other components useful, for example, for delivery, stability or activity of the conjugate. In this embodiment, a selected molecule of interest in
30 combination with Tat protein or a molecule of interest-Tat protein conjugate, such as a selected

WO 91/09958

PCT/US90/07607

-28-

oligonucleotide sequence-Tat protein conjugate, can be administered in sufficient quantity to result in entry into cells, particularly cell nuclei, and inhibition (reduction or elimination) of the causative agent (e.g., virus or bacterium) or

05 provision of a missing or defective protein.

Administration of a selected molecule of interest in combination with Tat protein or of a molecule of interest-Tat protein conjugate may be by a variety of routes. For example, administration may be by

10 injection, infusion or other parenteral routes (e.g., subcutaneous, intravenous, intramuscular, intrasternal and intracranial injection or infusion techniques). Similarly, administration may be topical (administered topically), that is, applied
15 locally to a particular part of the body (e.g., skin, lower intestinal tract, vaginally, rectally) where appropriate. For example, in the case of a papillomavirus infection, topical administration would be an appropriate mode of administration.

20 A selected molecule of interest in combination with Tat protein or a molecule of interest-Tat protein conjugate can also be used in making a vaccine. For example, the molecule of interest can be an antigen from the bacteria or virus or other
25 infectious agent that the vaccine is to immunize against (e.g., pgl20 of HIV). Providing the antigen into the cell cytoplasm allows the cell to process the molecule and express it on the cell surface. Expression of the antigen on the cell surface will

WO 91/09958

PCT/US90/07607

-29-

raise a killer T-lymphocyte response, thereby inducing immunity.

- For example, for in vivo applications, a selected conjugate can be formulated in appropriate compositions which include pharmacologically appropriate carriers, adjuvants and vehicles. In general, these carriers include aqueous or alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles can include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's or fixed oils. In addition, intravenous vehicles can include fluid and nutrient replenishers, and electrolyte replenishers, such as those based on Ringer's dextrose. Preservatives and other additives can also be present, such as, for example, antimicrobials, antioxidants, chelating agents, and inert gases. See, generally, Remington's Pharmaceutical Sciences, 16th Ed., Mack, ed. 1980. The amount of conjugate administered will vary and will depend on such factors as the condition or disease in question, the mode of administration, and the individual's size.

25 Examples

The subject invention will now be illustrated by the following examples, which are not to be seen as limiting in any way.

WO 91/09958

PCT/US90/07607

-30-

Example I Bacterial Expression and Purification of Tat

Two plasmids were constructed to produce the Tat protein in E. coli; one expresses amino acids 1-86 (the entire coding sequence) and the other expresses the first coding exon of Tat (residues 1-72). It is known that the second exon is not required for activity (Cullen, B.R., Cell, 46: 973-982 (1986); Muesing, M.A., et al., Cell, 48: 691-701 (1987); Sodroski, J., et al., Science, 229: 74-77 (1985); Frankel, A.D., et al., Proc. Natl. Acad. Sci., USA, 86:7397-7401 (1989)). Synthetic tat genes were constructed and ligated into the NdeI site of pET-3a, a plasmid that uses a strong bacteriophage T7 promoter to express cloned genes (Studier, F.W. and B.M. Moffat, J. Mol. Biol., 189: 113-130 (1986); Rosenberg, A.H., et al., Gene, 56: 125-135 (1987)). The resulting plasmids, ptat72 and ptat86, express Tat (residues 1-72 or 1-86, respectively) as 1%-5% of total E. coli protein. Both proteins gave similar results in all experiments. BL21(DE3) cells were used for expression and these cells also contained a plasmid expressing the T7 lysozyme gene to inhibit any T7 RNA polymerase expressed prior to induction (F.W. Studier, personal communication). Tat was induced with isopropyl β -D-thiogalactopyranoside (IPTG) (Studier, F.W. and B.M. Moffat, J. Mol. Biol., 189: 113-130 (1986)) and purified essentially as described (Frankel, A.D., et al., Science, 240: 70-73 (1988)) except that Tat was extracted from the

WO 91/09958

PCT/US90/07607

-31-

polyethyleneimine pellet with 10% ammonium sulfate instead of 700 mM KCl, and the S-Sepharose chromatography was eliminated.

Example II Synthetic Tat Peptides

05 Syntheses were performed using Fmoc chemistry on a Milligen/Bioscience model 9600 peptide synthesizer with a peptide amide linker-norleucine-4-methylbenzhydrylamine (PAL-Nle-MBHA) polystyrene resin (Milligen/Bioscience; 0.5 g). The benzotriazole-1-yl-oxytris(dimethylamino)phosphonium hexafluorophosphate/1-hydroxybenzotriazole (BOP/HOBt) coupling method (Hudson, D., J. Org. Chem., 53: 617-624 (1988)) was used with coupling times of 1-4 hours and with double coupling of His-33. Protecting groups were t-butyl ester (for Glu and Asp), 2,2,5,7,8-pentamethylchroman-6-sulfonyl (Arg), t-butyloxycarbonyl (Lys), trityl (His and Cys), t-butyl (Ser, Thr, and Tyr), and trimethoxybenzyl (Asn and Gln). All peptides were synthesized as their C-terminal amides. After synthesis was completed, protecting groups were removed and the peptide chains were cleaved from the resin with trifluoroacetic acid/ethanedithiol/thioanisole/anisole (90:3:5:2, vol/vol). The mixture was filtered and the products were obtained by addition of cold anhydrous diethyl ether to the filtrate. The precipitate was collected by filtration, thoroughly washed with ether and dried.

Peptides were treated with 0.5 M dithiothreitol at 37°C for 30 minutes to ensure complete reduction

WO 91/09958

PCT/US90/07607

-32-

of the cysteines and were purified on a C₄ HPLC column (Vydac) using an acetonitrile gradient in 0.1% trifluoroacetic acid. Amino acid composition was determined by hydrolysis in 6 M HCl containing 0.5% phenol at 100°C and analysis on a LKB Alpha Plus analyzer. Peptide purity (>90%) was determined by HPLC using an acetonitrile gradient of <0.5% per minute.

10 Example III Uptake of ¹²⁵I-Labeled Tat

Tat (residues 1-72) was labeled with ¹²⁵I by treating 500 µg of protein with 0.5 mCi ¹²⁵I and IODO-BEADS (Pierce) in 0.1 M Tris-HCl (pH 7.5) at room temperature for 5 minutes. The sample was dialyzed to remove unreacted ¹²⁵I. The specific activity was approximately 10⁶ cpm/µg protein. HL3T1 cells (2 x 10⁶ cells per dish) were treated with 5 µg radioactive Tat in the presence or absence of 100 µM chloroquine. Medium was removed at various times, cells were washed with PBS and EDTA, and cells were trypsinized for 10 minutes. Pancreatic trypsin inhibitor was added (5 µg/ml), cells were chilled to 4°C, centrifuged at 100Xg, and the supernatant was saved. The cell pellet was washed twice with serum-free DMEM, once with PBS and nuclei were isolated by lysis in 0.5% NP-40 as described (Ausubel, F.M. et al., Current Protocols in Molecular Biology (New York: John Wiley and Sons, 1987)). ¹²⁵I was counted using an LKB gamma counter.

WO 91/09958

PCT/US90/07607

-33-

Example IV Chloroquine Stimulated Tat Uptake

The parameters of chloroquine-stimulated Tat activity were studied in more detail. Figure 6A shows that the concentration dependence of chloroquine is a rather sharp dose response with maximum transactivation observed at 100 μ M chloroquine. This concentration is typically used to raise vacuolar pH (Mellman, I. et al., Annu. Rev. Biochem. 55:663-700 (1986)).

The time course of Tat transactivation in the presence of chloroquine showed a plateau after 24 hours (Figure 6B), and transactivation in the presence of chloroquine increased with increasing Tat concentration (Figure 6C). Transactivation was detectable with Tat concentrations as low as 1 nM.

Controls were done to determine whether transactivation was dependent on an intact TAR site, to determine whether a heterologous promoter could be stimulated by Tat, and to determine whether any of the effects seen with chloroquine occurred when Tat was produced intracellularly. After transient transfection of HeLa cells with an HIV-LTR plasmid (p-167/+80; Rosen, C.A. et al., Cell 41:813-823 (1985)), high levels of transactivation were seen when Tat was introduced by cotransfection with a Tat expression plasmid (pSV2tat72), by scrape-loading purified Tat, or by treatment with Tat and chloroquine. However, expression from the HIV-LTR containing a mutant TAR site (p-167/+21; Rosen, C.A. et al., Cell 41:813-823 (1985)) or from the SV40 early promoter (pSV2-CAT; Gorman, C.M. et al., Mol.

WO 91/09958

PCT/US90/07607

-34-

Cell. Biol. 2:1044-1051 (1982)) was not stimulated when Tat was introduced by these methods. Thus, introducing Tat by scrape-loading or by uptake with chloroquine appears to transactivate the HIV-LTR by the same mechanism that occurs when Tat is produced intracellularly. Chloroquine had no effect when Tat was produced intracellularly; chloroquine treatment of HL3T1 cells transiently transfected with pSV2tat72 showed no additional transactivation.

10

Example V RNA Isolation and Analysis

For the RNase protection experiment, total RNA was isolated by the hot acidic phenol method (Queen, C. and D. Baltimore, Cell 33:741-748 (1983)).

15 HIV-1-specific probes for all hybridizations were prepared by in vitro transcription (with α -³²P UTP) of an EcoRV-linearized plasmid containing the EcoRV (-120) to HindIII (+80) fragment of the viral LTR (cloned into the plasmid sp73; Promega). RNA probes

20 were purified on Sephadex G-50 spin columns (Boehringer-Mannheim).

RNase protection experiments were performed as described (Ausubel, F.M. et al., Current Protocols in Molecular Biology (New York: John Wiley and Sons, 1987)). Twenty μ g of cellular RNA were hybridized overnight with 5×10^5 cpm of the RNA probe at 38 °C in 40 μ l of 80% formamide, 40 mM PIPES (pH 6.7), 200 mM NaCl, 1 mM EDTA. Single-stranded RNA was

25 digested with RNase A (10 μ g/ml) and RNase T1 (45 U/ml) (Boehringer-Mannheim) in 400 μ l of 10 mM

30 Tris-HCl (pH 7.5), 300 mM NaCl, 5 mM EDTA for 1 hour

WO 91/09958

PCT/US90/07607

-35-

at room temperature. Protected fragments were analyzed by electrophoresis on 6% polyacrylamide-7M urea sequencing gels. Protected RNAs were visualized by autoradiography with intensifying screens and were quantitated using a Betascope 603 (Betagen).

Example VI Localization of Tat by Fluorescence

Purified Tat protein was labeled at lysine residues with tetramethyl rhodamine isothiocyanate (TRITC) by incubating 200 μ g of Tat (amino acids 1-72) in 0.1 M Na_2CO_3 pH9.0 with 5 μ g of TRITC dissolved in 5 μ l dimethylsulfoxide (DMSO), for 8 hours at 4 $^\circ\text{C}$. Unreacted TRITC was quenched with 50 mM NH_4Cl . The pH was lowered to 7.0 with HCl and rhodamine-labeled Tat was purified from free TRITC by dialysis against 50 mM Tris, pH 7, 1 mM DTT.

HL3T1 cells were grown on glass coverslips and incubated for various lengths of time with rhodamine-conjugated Tat (TRITC-Tat) in DMEM. H938 cells in suspension were incubated with rhodamine-conjugated Tat in RPMI. Cells were washed three times with phosphate buffered saline and viewed live on a Zeiss Axiophot fluorescence microscope.

Example VI Uptake of TatE2C Fusion Protein

Cell lines. The mouse embryo fibroblast cell line Balb/c 3T3 (clone A31; Aaronson and Todaro, J. Cell Physiol. 72:141-148 (1968)) was obtained from the American Type Culture Collection. HeLa cells were

WO 91/09958

PCT/US90/07607

-36-

obtained from Dr. Alan Frankel (Whitehead Institute, MIT). Both cell lines were propagated in Dulbecco's minimal essential medium (GIBCO) supplemented with 10% donor calf serum (Hazelton) and 4 mM glutamine (Whittaker). Cells were grown in a 5.5% CO₂ incubator at 37°C. Passaging of cells was performed by washing with phosphate-buffered saline and treating with trypsin (both GIBCO) to remove cells from plates followed by addition of culture medium and dilution of cells into plates containing fresh culture medium.

The HeLa cell line containing a Tat responsive reporter construct (HeLa.318) was generated by the introduction and stable selection of plasmid pXB318 (described below) by electroporation as described by Chu et al. (Chu et al., Nucleic Acids Res. 15:1311-1326 (1987)). pXB318 DNA was electroporated together with the selectable marker pSV2-neo (Southern, E.M. and Berg, P. J. Mol. Appl. Genet. 1:327-341 (1982)). Stable transfectants were selected in the presence of G418 (Southern, E.M. and Berg, P. J. Mol. Appl. Genet. 1:327-341 (1982)), and the presence of pXB318 DNA was confirmed by Southern blot hybridization analysis (Southern, E.M., J. Mol. Biol. 98:503-517 (1975)).

Vector Constructions. All molecular cloning reactions were carried out by methods described by Maniatis et al. (Maniatis, T., Fritsch, E.F., and Sambrook, J., Molecular Cloning: A Laboratory Manual (Cold Spring Harbor Laboratory, NY (1982)),

WO 91/09958

PCT/US90/07607

-37-

using enzymes obtained from New England Biolabs (Beverly, MA).

The TatE2C fusion protein (protein TatE2C), in which HIV Tat was fused to the carboxy terminal portion of BPV-1 E2, was expressed from the bacterial expression plasmid pFTE103. This plasmid was derived from ptat72 (see Example I) by insertion of a StyI-SpeI fragment which was isolated from vector pCO-E2 (Hawley-Nelson *et al.*, EMBO J. 7:525-531 (1988)) and which encodes the C-terminal portion of the E2 protein. Four synthetic deoxyoligonucleotides were used in the construction described below in detail.

The plasmid ptat72 was cleaved with the restriction endonucleases NdeI and BamHI releasing the Tat encoding portion of the vector. The 4603 base pair (bp) vector fragment was purified by agarose gel electrophoresis, and a 169 base pair (bp) NdeI-AatII fragment of the Tat encoding fragment was isolated. The 3' portion of the E2C coding sequence was isolated as a 375 bp StyI-SpeI fragment from pCO-E2 (Hawley-Nelson *et al.*, EMBO J. 7:525-531 (1988); obtained from Dr. Elliot Androphy, Tufts University/New England Medical Center Hospitals). The E2C fragment was connected to the Tat fragment and to the expression vector by use of two pairs of complementary deoxyoligonucleotides (synthesized according to standard procedures using an Applied Biosystems 380A DNA Synthesizer). One complementary pair of oligonucleotides was designed

WO 91/09958

PCT/US90/07607

-38-

to join the AatII overhang of the Tat fragment to the StyI overhang of the E2C fragment:

oligo 374-3

5' CGTCCGCCGCAGGGATCCCAGACCCACCAGGTTCCGGTTACTCTGC 3'
05 3' TGCAGCAGGCGGCGTCCCTAGGGTCTGGGTGGTCCAAGGCCAATGAGACGGTTC 5'

oligo 374-4

A second pair of complementary oligonucleotides was designed to link the SpeI overhang of the E2C fragment to the BamHI overhang of the 4603 bp vector backbone isolated from ptat72:

10

oligo 374-5

5' CTAGTGGCTCGAGATTCCG 3'
3' ACCGAGCTCTAAGGCCTAG 5'

oligo 374-6

15 The Tat fragment, the E2C fragment and the two pairs of oligos were inserted into the 4603 ptat72 vector backbone to create pFTE103. The resulting fusion gene is designed to express a protein comprising amino acids 1 through 67 of Tat at the amino
20 terminus followed by the C-terminal 105 amino acids of E2 (residues 306 through 410 of BPV01 E2).

The Tat responsive reporter construct pXB318 was constructed in three steps. The starting plasmid was pBG312 (Cate et al., Cell 45:685-698
25 (1986)). Two oligodeoxynucleotides were synthesized, which when annealed have an AatII-compatible overhang at the 5' end and an

WO 91/09958

PCT/US90/07607

-39-

EcoRI-compatible overhang at the 3' end, and form a polylinker with internal XhoI, HindIII and BamHI restriction sites:

5' CTCGAGAAGCTTGACGGATCCG 3'
05 3' TGCAGAGCTCTTCGAACTGCCTAGGCTTAA 5'

pBG312 was cleaved with AatII and EcoRI to remove the promoter, and the above polylinker was inserted into the vector to form the promoterless vector pXB100. The HIV-1 long terminal repeat (LTR) from
10 pU3R-III (Sodroski et al. Science 227:171-173 (1985)) was excised as a XhoI-HindIII fragment and was inserted into XhoI and HindIII sites of the polylinker of pXB100 to create pXB301. The human tissue plasminogen activator (tPA) cDNA reporter was
15 excised as a BamHI fragment from pTPA114 (Fisher et al. J. Biol. Chem. 260:11223-11230 (1985)) and inserted into the BamHI site of pXB301 to create pXB318.

Expression and purification of TatE2C. The TatE2C
20 fusion protein was expressed in E. coli using the vector pFTE103 and the T7 RNA polymerase expression system precisely as described by Studier et al. (Studier et al., Methods in Enzymology 185:60-89 (1990)).

25 Virtually all of the TatE2C protein was found in the insoluble fraction. The following purification was performed:

WO 91/09958

PCT/US90/07607

-40-

1. E. coli were pelleted, resuspended in ten packed cell volumes of 25 mM Tris-HCl pH 7.5, 1 mM EDTA, 10 mM DTT, and 1 mM PMSF and lysed with two passages through a French press.
- 05 2. The membrane fraction was pelleted by centrifugation at 10,000 rpm for 30 minutes.
3. This membrane fraction was resuspended in 6 M urea.
4. Solid guanidine-HCl was added to a final
- 10 concentration of 6 M and DTT was added to a final concentration of 10 mM.
5. After 30 minutes at 37°C, the solution was clarified by centrifugation at 10,000 rpm for 30 minutes.
- 15 6. The sample was loaded onto an A.5 agarose gel filtration column in 6 M guanidine-HCl, 50 mM sodium phosphate pH 5.4, and 10 mM DTT.
7. TatE2C-containing fractions were loaded onto a C₁₈ reverse phase HPLC column and eluted with a
- 20 gradient of 0-75% acetonitrile in 0.1% trifluoroacetic acid.

TatE2C protein appeared in a single peak. On protein gels, the TatE2C fusion protein migrated with an apparent molecular weight of 20,000 to

25 21,000 daltons.

Assay of TatE2C uptake by Tat activity. Uptake was detected either as Tat activity (activation of a Tat-dependent reporter in HeLa.318) or by indirect immunofluorescence using anti-E2 antibodies.

WO 91/09958

PCT/US90/07607

-41-

Tat activity was determined by adding Tat protein (amino acids 1-72) or Tate2C fusion protein at 2.5-250 nM along with chloroquine at 0.1 mM to the culture medium of HeLa.318 cells in 24 well plates essentially by the method of Frankel and Pabo (Frankel, A.D. and Pabo, C.O., Cell 55:1189-1193 (1988)). The culture medium was harvested 24 hours later and assayed for tPA activity by the method of Granelli-Piperno and Reich (J. Exp. Med. 148:223-234 (1978)). Cell numbers were determined and tPA secretion was expressed as ng/10⁶ cells per day. tPA secretion was undetectable in the absence of added Tat or Tate2C protein (less than 0.1 ng/10⁶ cells per day).

15 Assay of Tate2C uptake by E2-specific immunofluorescence. For indirect immunofluorescence, mouse 3T3 cells were seeded into LAB-TEK four chamber tissue culture chamber/slides. On the next day, Tate2C protein and chloroquine were added to the culture medium to final concentrations of 250 nM and 0.1 mM, respectively. Six hours later, immunofluorescence was performed as follows:

1. Medium was removed and wells were washed twice with phosphate-buffered saline (PBS).
- 25 2. Cells were fixed by treatment with 3.5% formaldehyde for 10 minutes at room temperature.
3. Cells were permeabilized in 0.2% Triton X-100/2% bovine serum albumin (BSA) in PBS with 1 mM

WO 91/09958

PCT/US90/07607

-42-

MgCl₂/0.1 mM CaCl₂ (PBS+) for 5 minutes at room temperature.

4. Cells were blocked by treatment with whole goat serum (Cappel #5506-1380) at a 1:30 dilution in
05 PBS+/2% BSA for one hour at 4°C.

5. The primary antibody was an affinity purified rabbit polyclonal which had been raised by injection of purified protein E2.C85 (in this case the carboxy terminal 85 amino acids expressed in bacteria using
10 the T7 polymerase expression system) into a rabbit, followed by purification by passage of the bleed over an E2 affinity column. This antibody was added to the wells at a 1:100 dilution in PBS+/2% BSA for one hour at 4°C.

15 6. The secondary antibody was a rhodamine conjugated goat anti-rabbit IgG (Cappel #2212-0081). This antibody was added at a 1:100 dilution in PBS+/0.2% BSA for 30 minutes at 4°C.

7. Wells were washed three times with PBS+/0.2%
20 Tween-20/2% BSA.

8. Slides were mounted in 50% glycerol in PBS and viewed with a fluorescent microscope with a rhodamine filter.

As a control, purified E2C protein (the carboxy
25 terminal 85 amino acids which were found to be recognized by the polyclonal antibody preparation) was added to wells in the same manner as the TatE2C fusion protein.

WO 91/09958

PCT/US90/07607

-43-

CLAIMS

1. A method of delivering a molecule of interest into a cell nucleus, comprising contacting a cell with a molecule of interest and Tat protein or a functionally effective fragment thereof, under conditions appropriate for uptake of Tat protein or a functionally effective fragment thereof into the cell.
05
2. The method of Claim 1 wherein the molecule of interest is selected from the group consisting of: proteins, peptides, oligonucleotides and drugs and the amino acid sequence of Tat protein in the molecule of interest-Tat protein conjugate is substantially the same as all or a portion of the amino acid sequence represented in Figure 1.
10
15
3. A method of delivering a molecule of interest into the nucleus of a cell, comprising the steps of:
20
 - a) providing a molecule of interest-Tat protein conjugate comprising:
 - 1) the molecule of interest and
 - 2) Tat protein or peptide having substantially the same amino acid sequence and substantially the same uptake activity as that of the
25

WO 91/09958

PCT/US90/07607

-44-

protein whose amino acid sequence is represented in Figure 1; and

- 05 b) contacting the molecule of interest-Tat protein conjugate with the cell in which delivery of the molecule of interest into the nucleus is desired, under conditions appropriate for uptake of HIV Tat protein into the cell.
- 10 4. The method of Claim 3 wherein the molecule of interest is selected from the group consisting of: proteins, peptides, oligonucleotides and drugs and the cell is present in cell culture medium.
- 15 5. The method of Claim 3 wherein the molecule of interest is selected from the group consisting of: proteins, peptides, oligonucleotides and drugs and the cell is present in blood obtained from an individual.
- 20 6. The method of Claim 5 wherein the molecule of interest is a protein or a peptide selected from the group consisting of: regulatory factors, antibodies, enzymes, toxins and drugs.
- 25 7. The method of Claim 4 wherein step (b) further comprises contacting the cell with a stabilizing agent, the stabilizing agent preventing proteolysis of the molecule of interest-Tat protein conjugate.

WO 91/09958

PCT/US90/07607

-45-

8. The method of claim 7 wherein the stabilizing agent is, for example, a metal or a lysomotrophic agent.
- 05 9. The method of Claim 8 wherein the lysosomotrophic agent is selected from the group consisting of:
 - a) chloroquine;
 - b) amantadine;
 - c) monensin;
 - 10 d) methylamine; and
 - e) ammonium chloride.
- 15 10. The method of Claim 4 wherein step (b) further comprises contacting the cell with a basic peptide, the peptide for example being Tat 38-58 or protamine.
- 20 11. A method of delivering a molecule of interest into a cell, comprising the steps of:
 - a) providing a molecule of interest-Tat protein conjugate comprising:
 - 20 1) the molecule of interest and
 - 2) Tat protein or peptide having substantially the same amino acid sequence and substantially the same uptake activity as that of the
 - 25 protein whose amino acid sequence is represented in Figure 1; and
 - b) contacting the molecule of interest-Tat protein conjugate with the cell in which

WO 91/09958

PCT/US90/07607

-46-

delivery of the molecule of interest is desired, under conditions appropriate for uptake of HIV Tat protein into the cell.

- 05 12. The method of Claim 11 wherein the molecule of interest is selected from the group consisting of: proteins, peptides, oligonucleotides and drugs and the cell is present in cell culture medium.
- 10 13. The method of Claim 11 wherein the molecule of interest is selected from the group consisting of: proteins, peptides, oligonucleotides and drugs and the cell is present in blood obtained from an individual.
- 15 14. The method of Claim 13 wherein the molecule of interest is a protein or a peptide selected from the group consisting of: regulatory factors, antibodies, enzymes, toxins and drugs.
- 20 15. The method of Claim 12 wherein step (b) further comprises contacting the cell with a stabilizing agent, the stabilizing agent preventing proteolysis of the molecule of interest-Tat protein conjugate.
- 25 16. The method of Claim 15 wherein the stabilizing agent is, for example, a metal or a lysomotropic agent.

WO 91/09958

PCT/US90/07607

-47-

17. The method of Claim 16 wherein the lysosomotropic agent is selected from the group consisting of:
- a) chloroquine;
 - b) amantadine;
 - c) monensin;
 - d) methylamine; and
 - e) ammonium chloride.
18. The method of Claim 12 wherein step (b) further comprises contacting the cell with a basic peptide, the peptide for example being Tat 38-58 or protamine.
19. A molecule of interest-Tat protein conjugate consisting essentially of:
- a) a molecule of interest selected from the group consisting of proteins, peptides, oligonucleotides and drugs; and
 - b) HIV Tat protein or a portion of the HIV Tat protein.
20. A molecule of interest-Tat protein conjugate consisting essentially of:
- a) a molecule of interest selected from the group consisting of proteins, peptides, oligonucleotides and drugs; and
 - b) HIV Tat protein or a portion of the HIV Tat protein wherein the HIV Tat protein has an amino acid sequence substantially

WO 91/09958

PCT/US90/07607

-48-

the same as all or a portion of the amino acid sequence represented in Figure 1.

21. A conjugate comprising a molecule of interest and Tat protein or a functionally effective fragment thereof for use as a medicament, e.g., under conditions appropriate for uptake of Tat protein or a functionally effective fragment thereof into a cell nucleus.
22. The conjugate of Claim 21 wherein the molecule of interest is selected from the group consisting of: proteins, peptides, oligonucleotides and drugs and the amino acid sequence of Tat protein in the molecule of interest-Tat protein conjugate is substantially the same as all or a portion of the amino acid sequence represented in Figure 1.
23. A conjugate comprising:
- 1) a molecule of interest and
 - 2) Tat protein or peptide having substantially the same amino acid sequence and substantially the same uptake activity as that of the protein whose amino acid sequence is represented in Figure 1;
- for use as a medicament, e.g., under conditions appropriate for uptake of HIV Tat protein into a cell upon contacting the conjugate with the

WO 91/09958

PCT/US90/07607

-49-

cell in which delivery of the molecule of interest into the nucleus is desired.

24. The conjugate of Claim 23 wherein the molecule of interest is selected from the group consisting of: proteins, peptides, oligonucleotides and drugs, for example a protein or a peptide selected from the group consisting of: regulatory factors, antibodies, enzymes, toxins and drugs.

25. A conjugate comprising:

- 1) a molecule of interest and
- 2) Tat protein or peptide having substantially the same amino acid sequence and substantially the same uptake activity as that of the protein whose amino acid sequence is represented in Figure 1;

- for use as a medicament, e.g., under conditions appropriate for uptake of HIV Tat protein into a cell upon contacting the conjugate with the cell in which delivery of the molecule of interest is desired.

26. The conjugate of Claim 25 wherein the molecule of interest is selected from the group consisting of: proteins, peptides, oligonucleotides and drugs, for example a protein or a peptide selected from the group consisting of:

WO 91/09958

PCT/US90/07607

-50-

regulatory factors, antibodies, enzymes, toxins and drugs.

27. Use of a molecule of interest-Tat protein conjugate consisting essentially of:
- 05 a) a molecule of interest selected from the group consisting of proteins, peptides, oligonucleotides and drugs; and
- b) HIV Tat protein or a portion of the HIV Tat protein wherein the HIV Tat protein
- 10 has an amino acid sequence substantially the same as all or a portion of the amino acid sequence represented in Figure 1, for the manufacture of a medicament for treatment or prophylaxis of a disease or
- 15 condition.
28. Use of a conjugate of a molecule of interest and Tat protein consisting essentially of:
- a) a molecule of interest selected from the group consisting of proteins, peptides,
- 20 oligonucleotides and drugs; and
- b) HIV Tat protein or a portion of the HIV Tat protein, for the manufacture of a medicament for treatment or prophylaxis of a disease or condition.
- 25 29. Use of a conjugate comprising a molecule of interest for delivery into a cell nucleus and Tat protein or a functionally effective fragment thereof, for the manufacture of a

WO 91/09958

PCT/US90/07607

-51-

medicament for administration under conditions appropriate for uptake of Tat protein or a functionally effective fragment thereof into the cell, for treatment or prophylaxis of a disease or condition.

05

30. The use of Claim 29 wherein the molecule of interest is selected from the group consisting of: proteins, peptides, oligonucleotides and drugs and the amino acid sequence of Tat protein in the molecule of interest-Tat protein conjugate is substantially the same as all or a portion of the amino acid sequence represented in Figure 1.

10

31. Use of a conjugate comprising:

15 a) a molecule of interest and
b) Tat protein or peptide having substantially the same amino acid sequence and substantially the same uptake activity as that of the protein whose amino acid

20 sequence is represented in Figure 1;
for the manufacture of a medicament for use for contacting the molecule of interest-Tat protein conjugate with cells in which delivery of the molecule of interest into the nucleus is

25 desired, under conditions appropriate for uptake of HIV Tat protein into the cell, for treatment or prophylaxis of a disease or condition.

WO 91/09958

PCT/US90/07607

-52-

32. The use of Claim 31 wherein the molecule of interest is selected from the group consisting of: proteins, peptides, oligonucleotides and drugs, for example a protein or a peptide
- 05 selected from the group consisting of: regulatory factors, antibodies, enzymes, toxins and drugs.
33. The use of Claim 32 wherein the medicament is administered topically.
- 10 34. The use of Claim 32 wherein the medicament further comprises a stabilizing agent, to prevent proteolysis of the molecule-Tat protein conjugate, the said agent for example being a metal or a lysosomotropic agent.
- 15 35. The use of Claim 34 wherein the lysosomotropic agent is selected from the group consisting of:
- a) chloroquine;
- b) amantadine;
- c) monensin;
- 20 d) methylamine; and
- e) ammonium chloride.
36. The use of Claim 32 wherein the medicament further comprises a basic peptide, the peptide for example being Tat 38-58 or protamine.
- 25 37. Use of a conjugate comprising:
- a) a molecule of interest and

WO 91/09958

PCT/US90/07607

-53-

- b) Tat protein or peptide having substantially the same amino acid sequence and substantially the same uptake activity as that of the protein whose amino acid sequence is represented in Figure 1;
05 for the manufacture of a medicament for use for contacting the molecule of interest-Tat protein conjugate with cells in which delivery of the molecule of interest is desired, under
10 conditions appropriate for uptake of HIV Tat protein into the cell, for treatment or prophylaxis of a disease or condition.
38. The use of Claim 37 wherein the molecule of interest is selected from the group consisting of:
15 of: proteins, peptides, oligonucleotides and drugs, for example a protein or a peptide selected from the group consisting of: regulatory factors, antibodies, enzymes, toxins and drugs.
- 20 39. The use of Claim 38 wherein the conjugate further comprises a stabilizing agent, to prevent proteolysis of the molecule of interest-Tat protein conjugate, the said agent for example being a metal or a lysosomotropic agent.
25
40. The use of Claim 39 wherein the lysosomotropic agent is selected from the group consisting of:
a) chloroquine;

WO 91/09958

PCT/US90/07607

-54-

- b) amantadine;
- c) monensin;
- d) methylamine; and
- e) ammonium chloride.

05 41. The use of Claim 38 wherein the medicament further comprises a basic peptide, the peptide for example being Tat 38-58 or protamine.

WO 91/09958

PCT/US90/07607

1 / 10

FIGURE 1

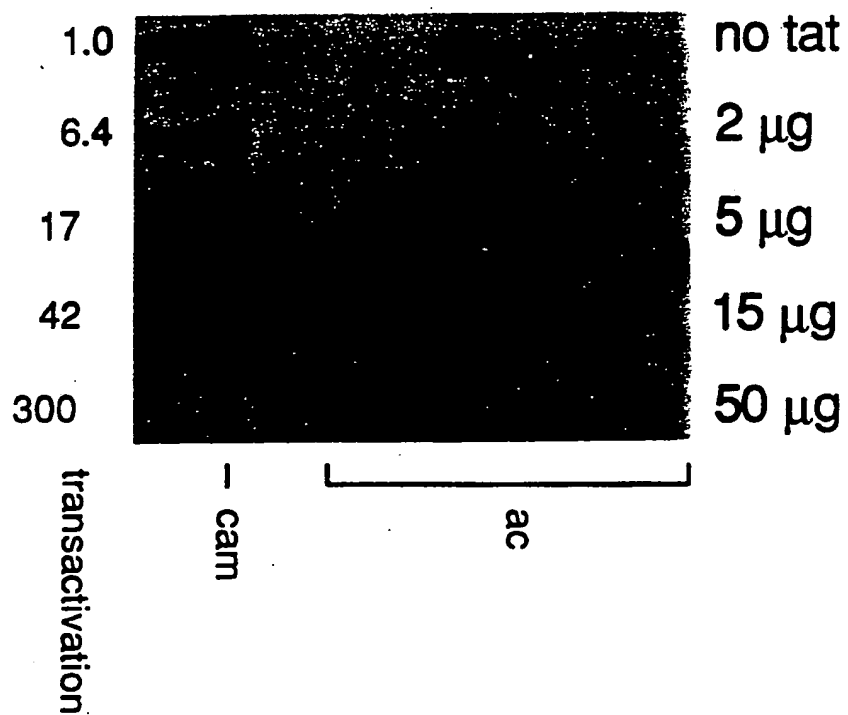
Met Glu Pro Val Asp Pro Arg Leu Glu Pro Trp Lys His Pro Gly Ser
 1 5 10 15
 Gln Pro Lys Thr Ala Cys Thr Asn Cys Tyr Cys Lys Lys Cys Cys Phe
 20 25 30
 His Cys Gln Val Cys Phe Ile Thr Lys Ala Leu Gly Ile Ser Tyr Gly
 35 40 45
 Arg Lys Lys Arg Arg Gln Arg Arg Pro Pro Gln Gly Ser Gln Thr
 50 55 60
 His Gln Val Ser Leu Ser Lys Gln Pro Thr Ser Gln Ser Arg Gly Asp
 65 70 75 80
 Pro Thr Gly Pro Lys Glu
 85

WO 91/09958

PCT/US90/07607

2 / 10

FIG.2

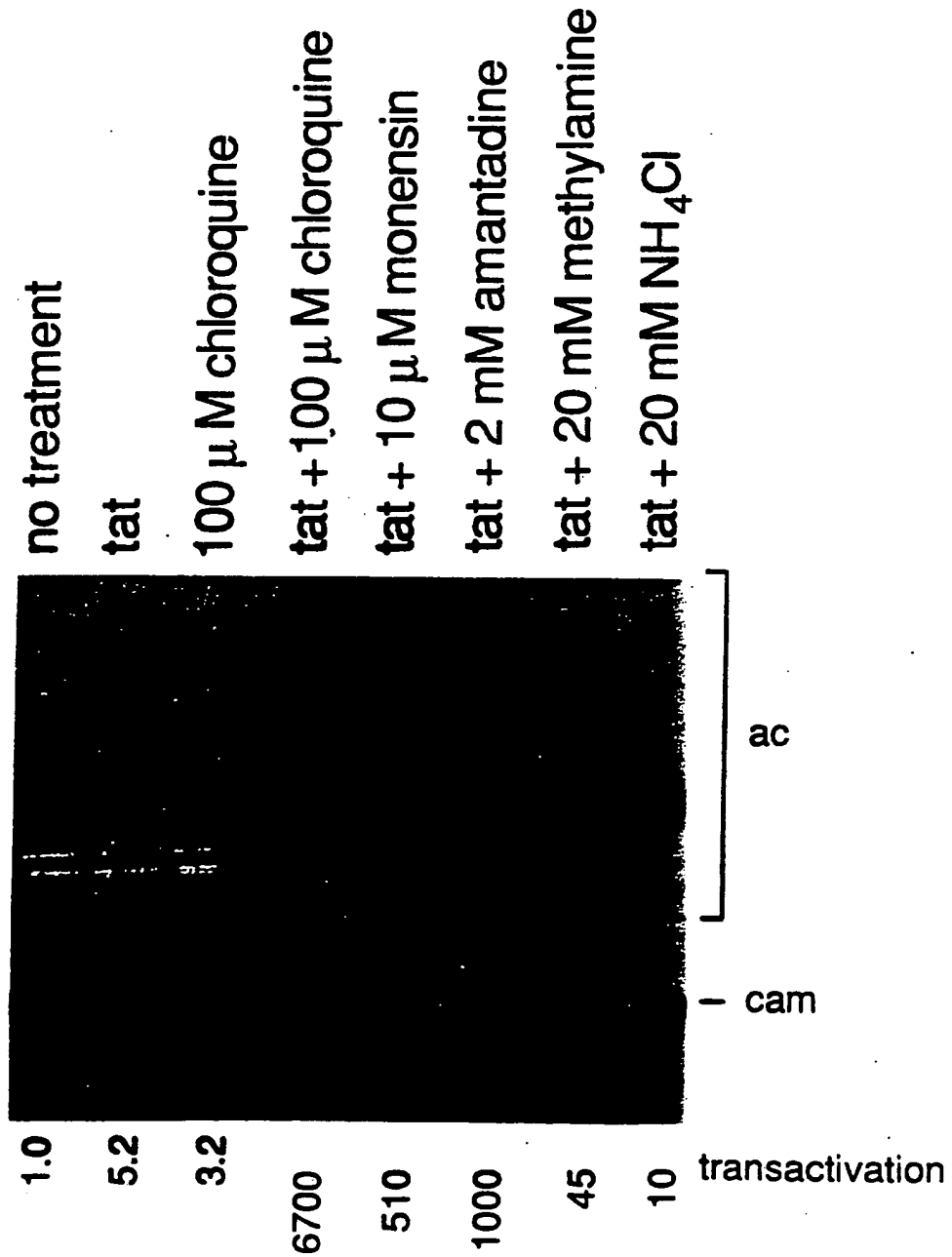


WO 91/09958

PCT/US90/07607

3 / 10

FIG.3



WO 91/09958

PCT/US90/07607

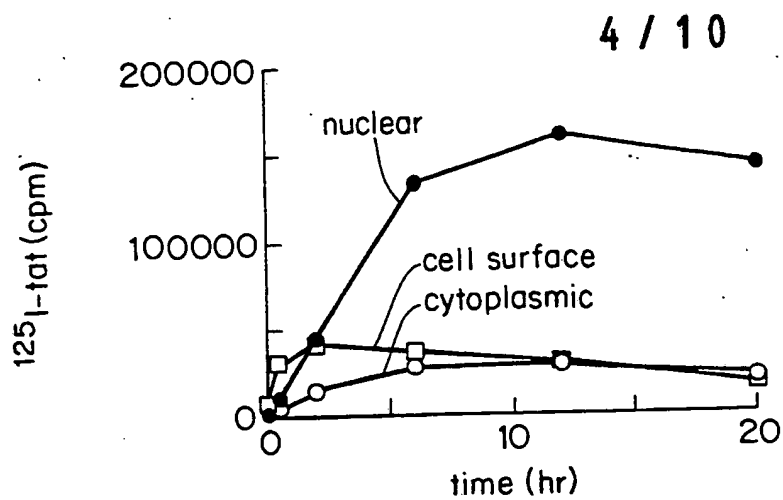


Fig. 4

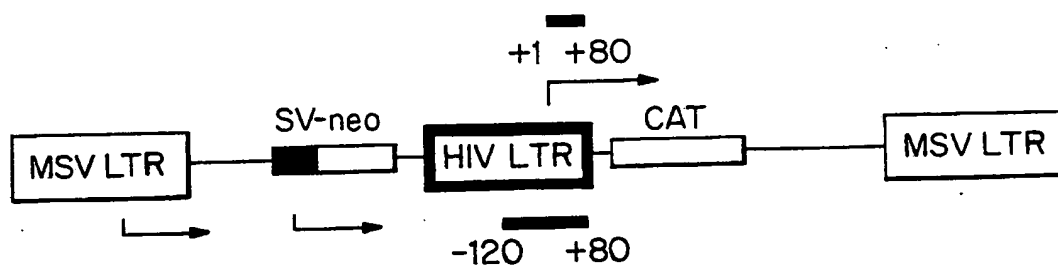


Fig. 9

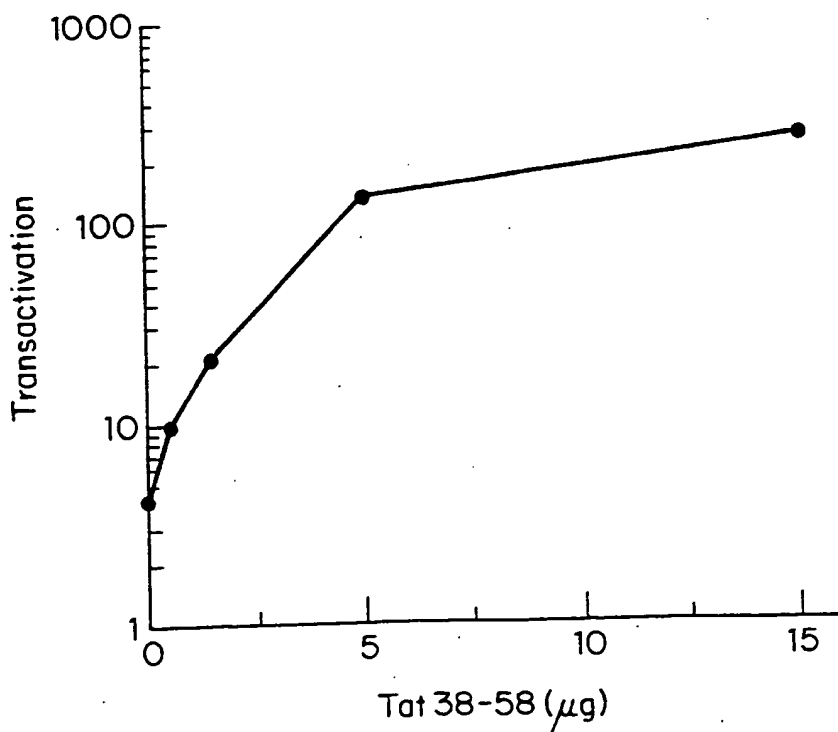


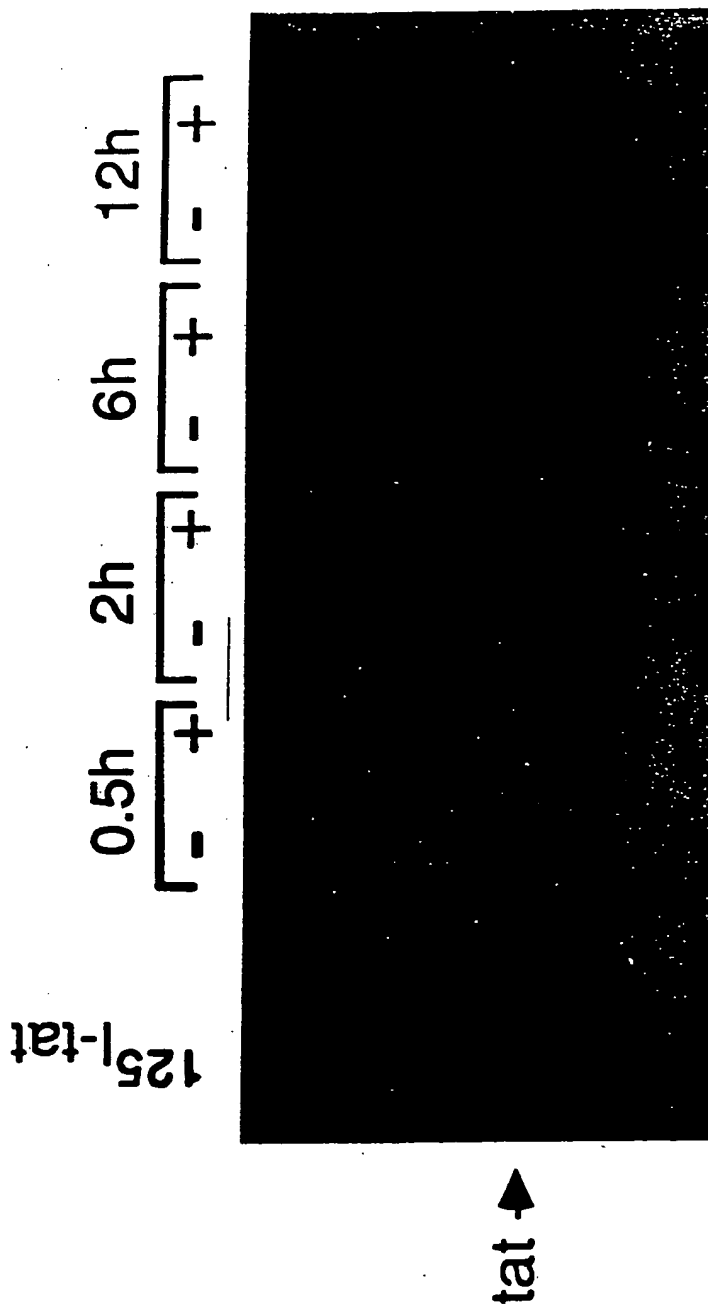
Fig. 11

WO 91/09958

PCT/US90/07607

5 / 10

FIG. 5



WO 91/09958

PCT/US90/07607

6 / 10

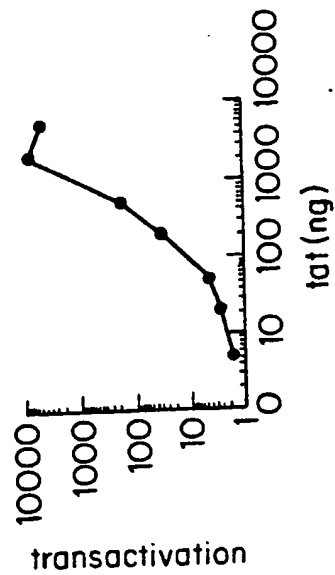


Fig. 6A

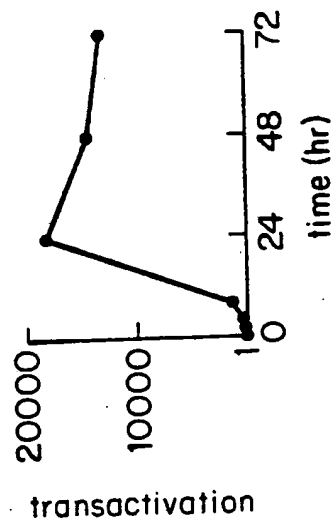


Fig. 6B

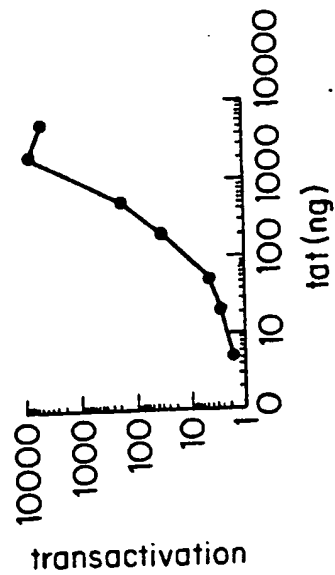


Fig. 6C

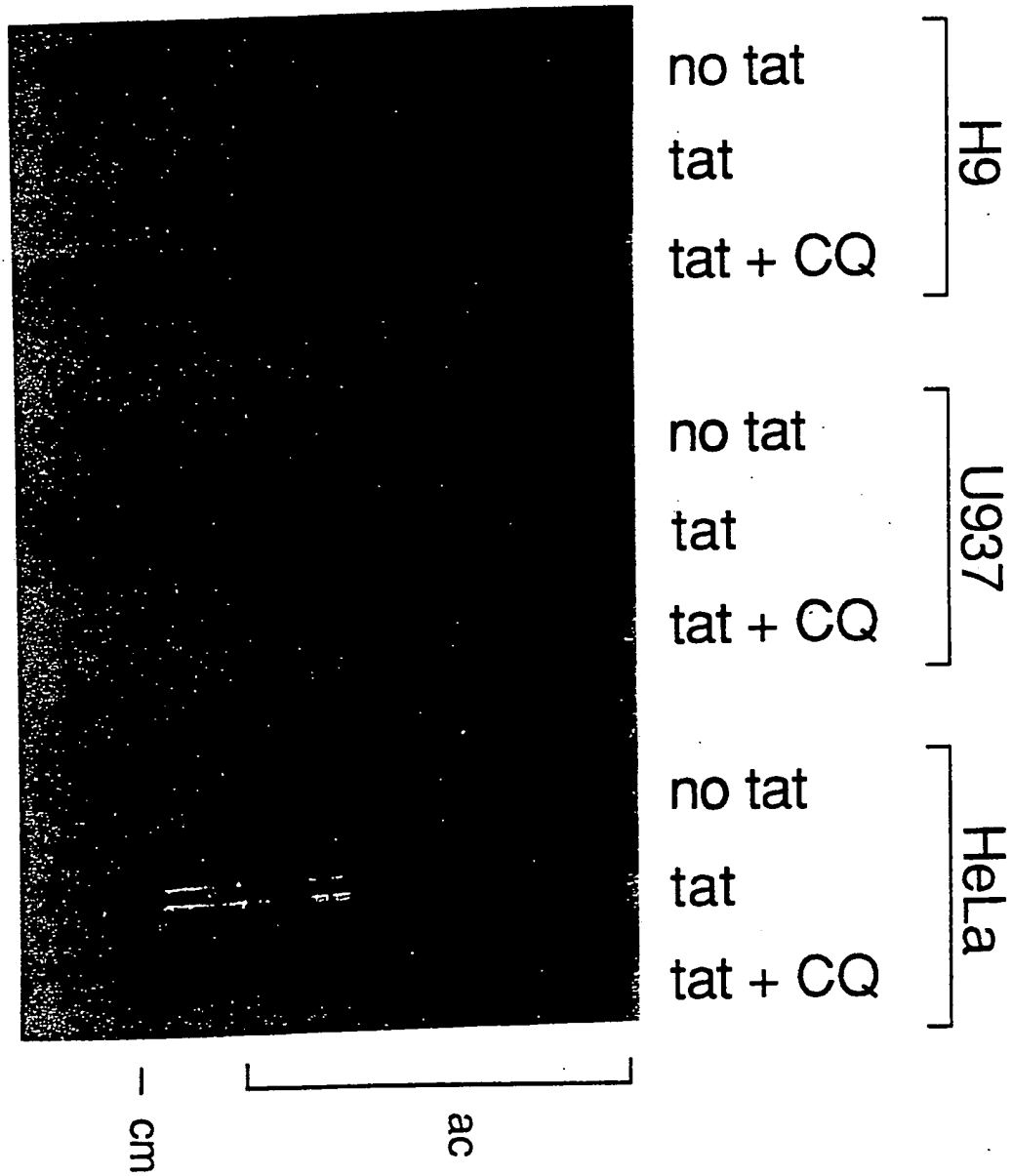
SUBSTITUTE SHEET

WO 91/09958

PCT/US90/07607

7 / 10

FIG.7

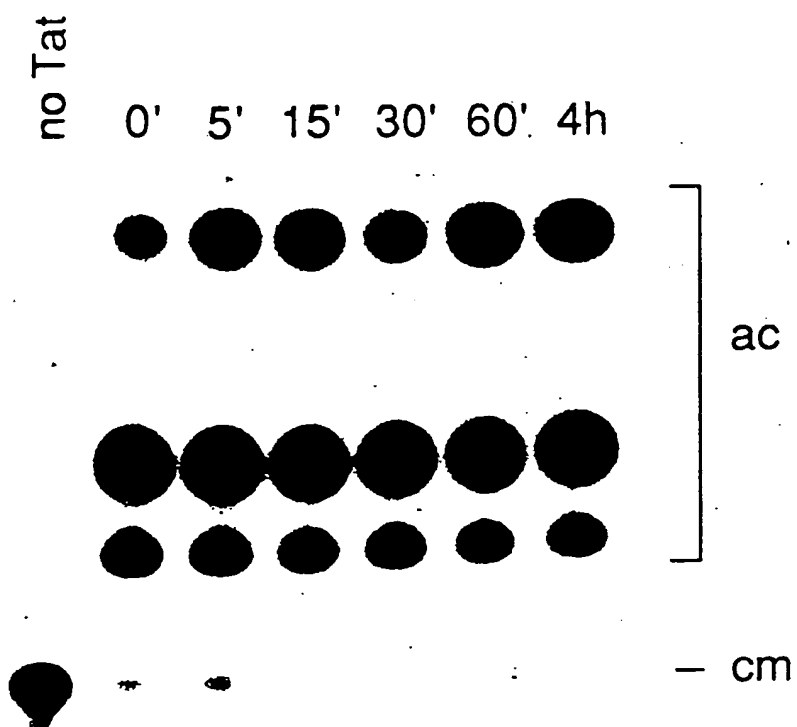


WO 91/09958

PCT/US90/07607

8 / 10

FIG. 8

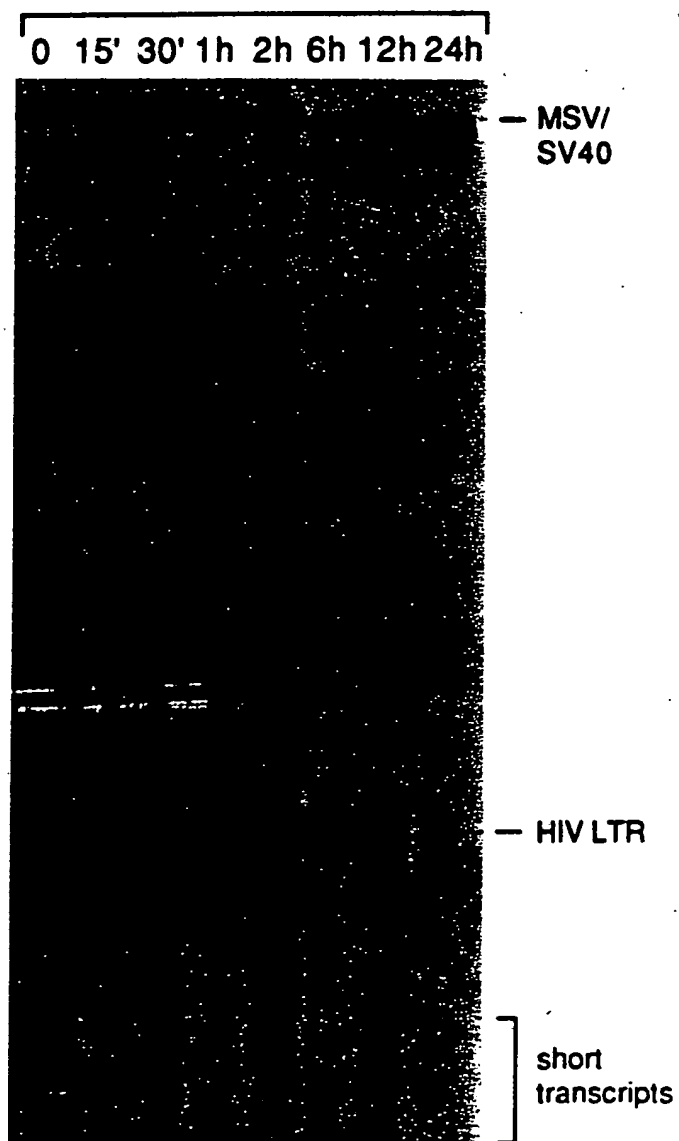


WO 91/09958

PCT/US90/07607

9 / 10

FIG.10



WO 91/09958

PCT/US90/07607

10 / 10

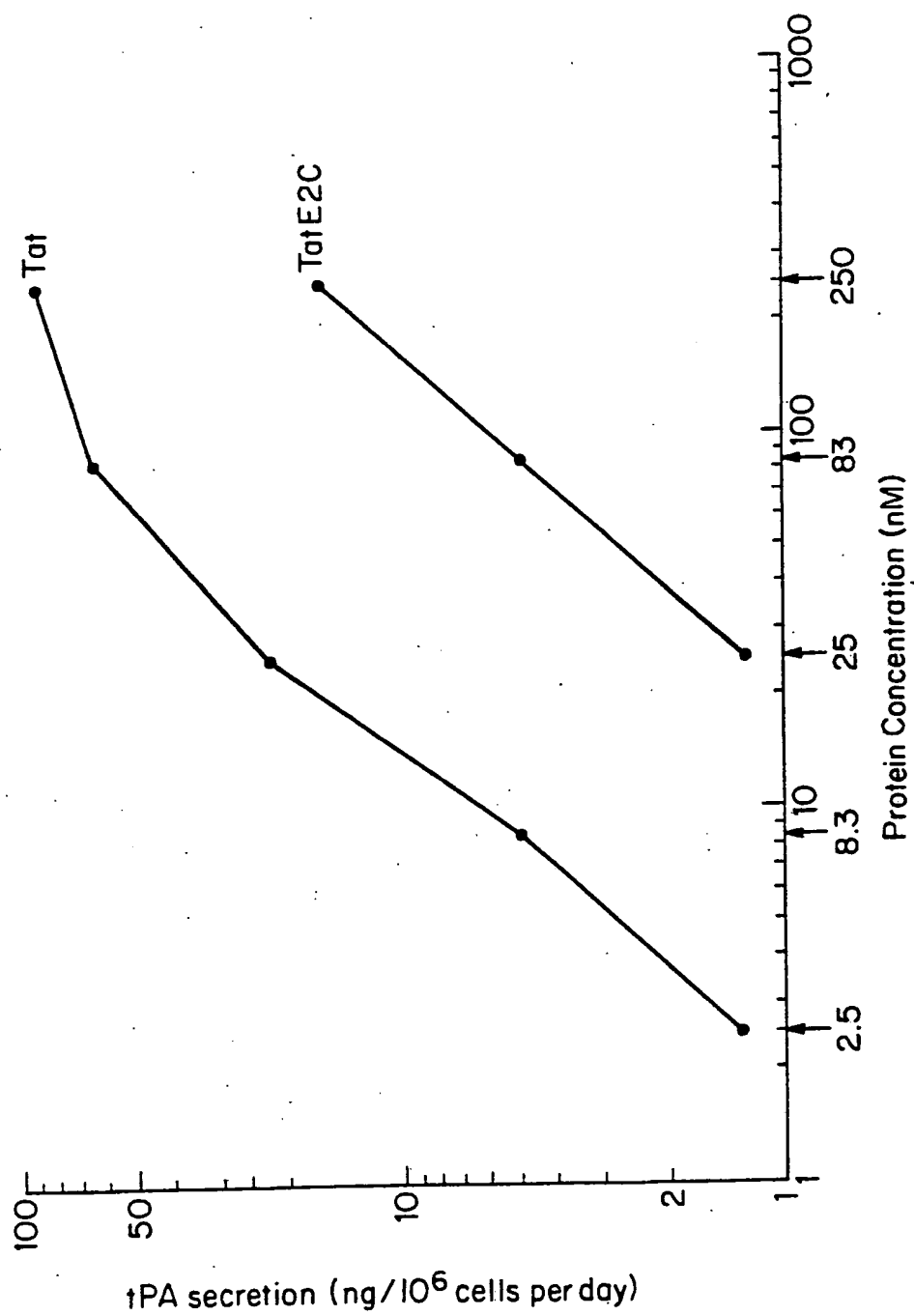


Fig. 12

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☐ FADED TEXT OR DRAWING
- ☐ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☒ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☐ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.